Microtubule formation and the initial association of tubulin dimers

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Microtubule protein could be prepared in high yield, and could form copious microtubules, in solutions containing glutamate but not in solutions containing only phosphate ions. Correspondingly, tubulin after isolation showed an association equilibrium in the presence of glutamate (or other zwitterions), but not in phosphate buffers. The correlation suggests that this association to tetramers is probably the initial step in the mechanism of microtubule formation.

Microtubule *Tubulin association* *Glutamate* *(Bovine brain)*

1. INTRODUCTION

The polymerization of tubulin to form microtubules depends on endogenous agents known as microtubule-associated proteins (MAPs), and polymerization can be enhanced by exogenous agents such as taxol [1], dimethyl sulphoxide (DMSO) [2], or glycerol [3]. However, microtubule formation also appears to be critically dependent on the solvent conditions as, in the absence of exogenous agents, microtubule formation occurs only in a limited range of buffer solutions. Thus, besides the importance of MAPs, it is necessary to explain the dependence of microtubule formation on these buffers and to investigate their effect on tubulin itself. This paper presents a correlation between the yields (and assembly) of microtubule proteins prepared in particular buffers and the association behaviour of pure tubulin in similar solutions.

Dedication: We should like to dedicate this communication to Professor S.P. Datta on the occasion of his retirement as Managing Editor of FEBS Letters. It is a pleasure to acknowledge the helpful support and encouragement that Prakash has given his colleagues, and in particular the kindness and friendship enjoyed over a long period by one of us (M.A.R.)

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2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Microtubule protein was prepared from bovine brain by cycles of temperature dependent assembly and disassembly [4]. Twice-cycled protein was routinely used for subsequent experiments.

Tubulin, free of MAPs, was prepared by chromatography on Whatman P-11 phosphocellulose [5]. The phosphocellulose was routinely pre-saturated with magnesium to ensure constancy of the ion concentration during the isolation of tubulin [6]. The column buffer was 0.02 M sodium phosphate, 0.1 M sodium glutamate, 2 mM EGTA, 2 mM DTT, 1 mM $MgSO_4$ and 0.1 mM GTP, pH 6.75. This mixture is subsequently referred to as phosphate-glutamate or PG buffer.

2.2. *Protein assay*

Protein determinations were made according to the Coomassie blue dye-binding method of Bradford [7] using bovine serum albumin for standardization.

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2.3. *SDS-polyacrylamide gel electrophoresis*

Electrophoresis on polyacrylamide in the presence of SDS was performed according to Laemmli [8]. Gels were stained with Coomassie blue and the bands quantitated by densitometry.

2.4. *Colchicine-binding assay*

The colchicine-binding activities of tubulin preparations were measured according to Sherline et al. [9]. Tritiated (*ring C methoxy*) colchicine was obtained from Amersham International. Free and bound colchicine were separated using activated charcoal (Sigma, London).

2.5. *Polymerization assay*

Microtubule assembly was induced by incubation of samples at 30°C and the addition of 1 mM GTP. The extent of microtubule formation was assessed by measurement of turbidity at 350 nm [10]. The morphology of the assembly products was checked by electron microscopy of negatively stained samples.

2.6. *Analytical ultracentrifugation*

Sedimentation velocity experiments were made at a speed of 59780 rpm, using a Beckman model E ultracentrifuge. The temperature was maintained at 5°C with an RTIC unit.

The correction factors for the density and viscosity of the various buffers used in this study were, where possible, calculated from values in International Critical Tables. Otherwise we used the sedimentation behaviour of bovine serum albumin to estimate the required correction factors. This relies on the similarity of the partial specific volume of bovine serum albumin, 0.734 ml/g [11], and that measured for tubulin, 0.736 ml/g [12]. The correction factors for the buffers at 5°C to water at 20°C are:

Phosphate-glutamate	1.701
Pipes (0.1 M)	1.791
Mes (0.1 M)	1.656
Sodium phosphate (0.05 M)	1.475 (1.565 calc.)
Sodium phosphate (0.07 M)	1.656 (1.595 calc.)

To minimize denaturation of tubulin arising from prolonged dialysis, protein samples were transferred to the various buffers by a 3–4-fold dilution followed by dialysis in the buffer for 4 h at 4°C.

3. RESULTS AND DISCUSSION

3.1. *Preparation of microtubule protein*

In PG buffer the yields of microtubule protein prepared by cycles of temperature-dependent assembly and disassembly were consistently good. Approx. 10% of the total soluble protein in the extract was recovered after 2 cycles of assembly. In contrast, the same procedure in phosphate buffer resulted in the much lower yield of 4% of the total soluble protein (table 1). The differences between the 2 buffers became more apparent with increasing cycles of assembly, the yield of microtubule protein being lower at each stage in phosphate buffer compared with that in PG buffer. This effect is also shown by successive turbidity assays where the competence to form microtubules increases in PG buffer but is virtually absent after 2 assembly cycles in phosphate buffer (fig.1). Additionally, estimates of colchicine binding by the 2 prepara-

Table 1

Relative yields of microtubule protein prepared in phosphate-glutamate or in phosphate buffers

Step	Phosphate-glutamate buffer		Phosphate buffer	
	Total protein (mg)	Yield (%)	Total protein (mg)	Yield (%)
Crude extract	4260	100	4460	100
C_1S	680	16.0	646	14.5
C_2S	406	9.5	169	3.8

C_1S and C_2S are the first and second 'cold supernatants' after disassembly of microtubules at low temperature

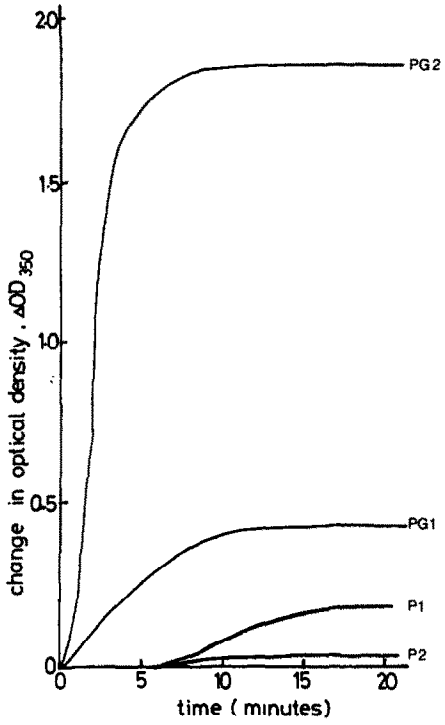

Fig.1. Assay of turbidity of samples of microtubule protein. PG1 and PG2 refer to the turbidity following incubation of C_1S and C_2S samples, respectively, during microtubule preparation in phosphate-glutamate buffer; P1 and P2 refer to the turbidity of the corresponding C_1S and C_2S samples during microtubule preparation in phosphate buffer. The protein concentration was standardized at 7 mg/ml for each experiment.

tions support the observation that microtubule protein prepared in PG buffer remains competent to form microtubules while that prepared in phosphate buffer has virtually lost this ability. Thus, after the second depolymerization (C_2S), the material prepared in PG buffer bound 0.45 ± 0.05 mol colchicine per mol tubulin, while corresponding material prepared in phosphate buffer only bound 0.07 ± 0.01 mol colchicine per mol tubulin.

These yields are not related to differences in composition of the microtubule protein as electrophoresis on SDS-polyacrylamide shows that microtubules prepared in PG buffer or in phosphate buffer have the same MAP content (0.02 ± 0.004 and 0.09 ± 0.01 for the molar ratio of MAP1 and MAP2, respectively, to tubulin).

3.2. *Sedimentation behaviour*

In PG buffer microtubule protein gave the characteristic profile (fig.2a) also seen in Pipes buffer [13], with a small proportion of free tubulin (6 S) and particular oligomers referred to as 18 S and 30 S species [13]. A different pattern of smaller oligomers was seen in phosphate buffers and a larger proportion of the protein remained as free tubulin (fig.2b). Sedimentation of pure

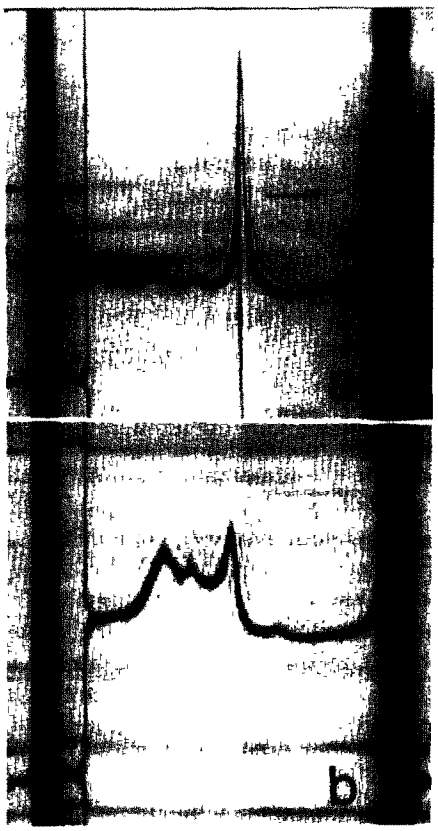

Fig.2. Comparison of oligomer formation by microtubule protein in phosphate-glutamate and in phosphate buffers. (a) Sedimentation in phosphate-glutamate of 14 mg/ml microtubule protein, with the schlieren plate angle set at 60°. (b) Sedimentation in phosphate buffer of 10 mg/ml microtubule protein, with the schlieren plate angle set at 45°. Both solutions were at pH 6.75 and contained 2 mM EDTA, 2 mM DTT, 1 mM $MgSO_4$ and 0.1 mM GTP. The temperature was 5°C. The photographs were taken 40 min after reaching a speed of 59780 rpm. In both patterns the 6 S component is the slowest one present, but it forms a much larger proportion of the protein in the second example.

tubulin gave schlieren patterns of the type shown in fig.3. In buffer containing phosphate alone (fig.3d), a single symmetrical boundary was observed confirming the homogeneity of the tubulin preparation. However, in the various zwitterionic buffers (fig.3a–c), preparations of tubulin consistently showed a skewed boundary, the extent of skewing increasing with protein concentration. The sedimentation coefficient of the main component of the boundary also increased with increasing protein concentration (fig.4). The increase was steep at lower protein concentrations and tended to a plateau at higher protein concentrations. In phosphate buffer no effect of increased protein concentration on the sedimentation of tubulin was observed. The asymmetry of the boundary and the

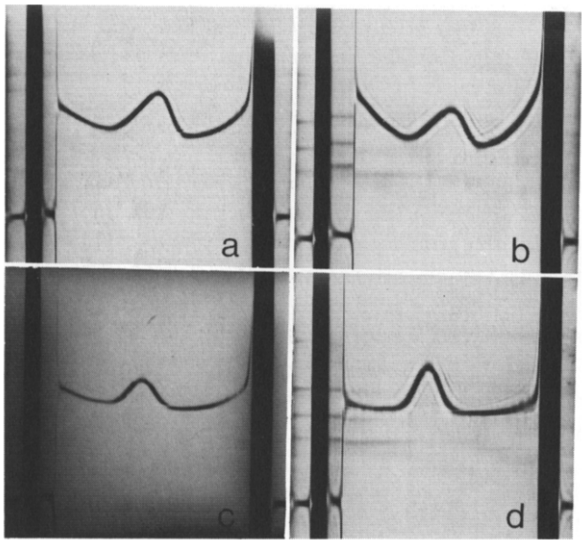

Fig.3. Sedimentation patterns of tubulin in various buffer solutions. (a) Sedimentation in phosphate-glutamate of 9 mg/ml tubulin with the schlieren plate angle set at 50°. (b) Sedimentation in 0.1 M Pipes of 9 mg/ml tubulin with the schlieren plate angle set at 45°. (c) Sedimentation in 0.1 M Mes of 6 mg/ml tubulin with the schlieren plate angle set at 45°. (d) Sedimentation in phosphate buffer of 8 mg/ml tubulin with the schlieren plate angle set at 40°. The photographs were taken 90 min after reaching a speed of 59780 rpm. Other conditions were as given in the legend to fig.2. The patterns in a and b show pronounced skewing of the boundary. Skewing is less evident in c where the protein concentration is reduced; and is absent in d, where the symmetrical boundary indicates the presence of a discrete molecular entity in this solution.

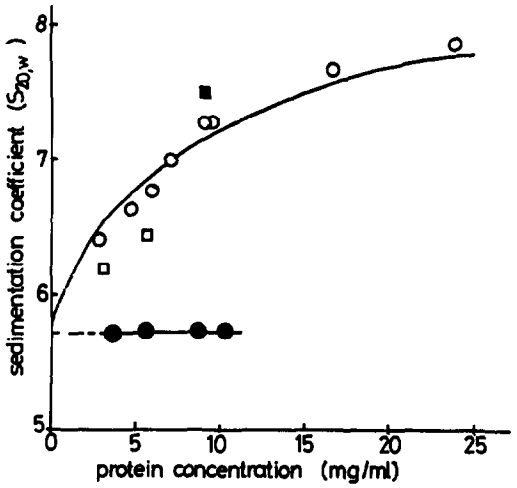

Fig.4. Dependence of sedimentation coefficient on the concentration of tubulin. Sedimentation was carried out in phosphate-glutamate (○), Pipes (■), Mes (□) and phosphate (●). Experimental conditions were as given in the legend to fig.3. The curve was calculated for a dissociation constant of 3.8×10^{-4} M and the relation between the sedimentation coefficients for tetramer and dimer given in the text.

observed variation in its mobility suggest an association equilibrium occurring in the zwitterionic buffers but absent in phosphate buffer. The equilibrium can be quantitated in terms of a dissociation constant for a tetrameric species of tubulin:

$$K = \frac{[\alpha\beta]^2}{[\alpha_2\beta_2]}$$

which may be expressed on a weight basis:

$$K = \frac{\phi^2}{(1-\phi)} \cdot \frac{2c}{M}$$

where ϕ is the degree of dissociation on a weight basis, c is the total protein concentration in g/l, and M is the relative molecular mass of the tubulin dimer, namely 110 kDa [14].

The parameter ϕ is related to the sedimentation data by [15–17]:

$$\phi = \frac{2(s_2 - s_f)}{(s_2 - s_f) + (s_2 - s_1)}$$

Table 2

Estimate of the equilibrium constant for the dissociation of tubulin tetramers to dimers

Protein concentration (mg/ml)	s_f (S)	ϕ	K (10^{-4} M)
2.8	6.4	0.90	4.1
4.85	6.6	0.86	4.5
6.0	6.7	0.84	4.8
7.1	7.0	0.79	3.8
9.0	7.3	0.72	3.0
9.5	7.3	0.72	3.2
17.0	7.7	0.62	4.1
24.0	7.8	0.57	3.3
		mean	3.8 ± 0.7

where s_1 is the sedimentation coefficient of the dimer, s_2 that of the tetramer, and s_f that of the main component of the skewed boundary.

The results gave 5.8 S for the value of s_1, and on the basis of an association of 2 such units a value of 9.2 S would be expected for s_2. Using the observed values of s_f, the dissociation constant for the tetramer-dimer equilibrium is about 4×10^{-4} M (table 1).

3.3. *Comparison with previous work*

The presence of tetramers has been invoked to explain the equilibrium concentration distribution in studying the dissociation of tubulin dimers to monomers [18].

Although the present account proposes an association to a tetrameric species with an anticipated $s_{20,w}$ near 9 S, the phenomenon is to be distinguished from that giving a 9 S species either by interaction with vinblastine [19], or by storage of tubulin solutions for several hours [20]. In these reports the 9 S entity clearly separated from 6 S tubulin dimers; the sedimentation boundaries did not conform to an interacting system, and association occurred with loss of assembly properties of tubulin [20].

In contrast, in the present work a discrete tetrameric species does not separate, but its presence is indicated by the dependence of sedimentation coefficient on concentration; the sedimenting pattern does conform to an interaction boundary, and there is a positive correlation between this equilibrium and the assembly properties, as shown by the retention of colchicine binding by tubulin, and by polymerization of microtubule protein in the same solutions. However, the present results support the contention that there is an absence of an interacting boundary and a concomitant loss in assembly properties in phosphate buffers, at concentrations of 0.05 M in this work and 0.01 M in the previous report [20].

Previous studies have been made on the effect of high concentrations (1 M) of multisite ions on polymerization of tubulin [21–24]. Such concentrations afford a means of isolating tubulin [21], but the polymers formed did not necessarily have the morphology of native microtubules. Thus, while smooth-walled microtubules formed in 1.8 M Mes, aberrant structures were produced in 0.8 M Pipes and in 1 M glutamate [22,23]. The polymerization was independent of MAPs, which also contrasts with the native property. Furthermore, the high ion concentrations approximate to conditions that influence the general solubility of proteins. Glutamate and Pipes have been shown to cause preferential hydration of tubulin which could promote its polymerization [24]. However, this polymerization does not necessarily give microtubules, as preferential hydration is non-specific and occurs with other proteins [24]. Therefore, it is not surprising that tubulin formed aberrant polymers in high concentrations of these ions [22,23].

In contrast, the ion concentrations were kept low (0.1 M) in the experiments described here. Of these ions, glutamate is the most relevant since its concentration in brain (10 mM) is high for a metabolite [25,26]. This average value implies higher localised concentrations, so that the level of 100 mM used in these experiments is not too extreme. Also, this ion concentration does not remove the need for control by MAPs on genuine microtubule formation, which would occur after the initial stages of tubulin self-association reported here.

Studies of near-UV circular dichroism indicate that removal of MAPs by phosphocellulose [6] yields tubulin which retains the conformation that it has in the native microtubule protein complex [27]. Dissimilar conformations can result from other methods of isolating tubulin [28], which

presumably assembles to alternative microtubule structures [27]. The preservation of a subtle conformational property, noted under certain conditions in the present work, may also depend on the use of the same phosphocellulose treatment [6] for removal of MAPs in the preparation of tubulin.

3.4. *Conclusions*

This work confirms the successful isolation of microtubule protein by cycles of assembly and disassembly in zwitterionic buffers and the inability to do so in phosphate buffers [29]. Poor microtubule assembly in phosphate buffer was confirmed by the dramatic loss of ability to polymerize as shown by the diminished development of turbidity (fig.1) and by the diminished colchicine binding given above. The conditions used for the isolation avoid all extraneous additions to promote microtubule assembly, so that the association phenomenon relies upon the MAP-tubulin and tubulin-tubulin interactions prevailing in the specified buffers.

In the absence of MAPs, the pure tubulin preparation appears to be a homogeneous entity (namely the tubulin dimer) in phosphate buffers, and exists in an association equilibrium (dimer to tetramer) in the zwitterionic buffers. The consistent correlation between this association and the solvent conditions which, in the presence of MAPs, favour microtubule formation suggests that these particular buffers in fact promote a tubulin-tubulin interaction which is absent in the phosphate buffer. The correlation also suggests that this initial self-association of tubulin is a prerequisite for the further polymerization to microtubules, a process which may then be modulated or accentuated by the MAPs.

Zwitterionic components such as Pipes, Mes and glutamate have been widely used in microtubule assembly studies [4,21–23,30] following the initial observations of Weissenberg [29] who found that microtubule formation and stability was increased in such solutions, with a concomitant decrease in aberrant products of assembly. This study demonstrates a possible physical basis for this effect, as stabilization of the tetrameric form of tubulin by such buffers could be the initial step in the mechanism of microtubule formation, which would improve the yield and produce the observed increase in stability of the resultant microtubules.

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